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Abstract: Aims: Effective stroke treatments beyond reperfusion remain scant. The natural steroid hormone progesterone has shown protective effects in experimental models of brain injury and cardiovascular disease. However, unfavorable bioavailability limits its clinical use. Desogestrel and drospirenone are new generation progestins with progesterone-like properties, developed as oral contraceptives with excellent bioavailability and safety profile. We investigated the neuroprotective properties of these progestins in vivo using transient middle cerebral artery occlusion (MCAO) and in vitro using an oxygen-glucose deprivation and reoxygenation (OGD/R) model in primary neuronal cells. Methods and Results: MCAO was induced in female, female ovariectomized (modeling postmenopausal females) and male mice. Treatment with the progestins resulted in less severe strokes after MCAO and less neuronal death in OGD/R. Desogestrel and drospirenone induced higher expression levels of GABAAR 4 and delta subunits within the brain, suggesting changes in GABAAR configuration favoring tonic inhibition as potential mechanism of action. Treatment with the GABAAR blocker picrotoxin abolished the protection afforded by the progestins in vivo and in vitro. Conclusions: For the first time, here we delineate a potential role of desogestrel and drospirenone, both clinically approved and safe drugs in mitigating the consequences of stroke. Contraception with desogestrel and drospirenone in progestin-only preparations may be particularly beneficial for women at risk of stroke.

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Contraceptive drugs mitigate experimental stroke-induced brain injury

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Abstract

Aims. Effective stroke treatments beyond reperfusion remain scant. The natural steroid hormone progesterone has shown protective effects in experimental models of brain injury and cardiovascular disease. However, unfavorable bioavailability limits its clinical use. Desogestrel and drospirenone are new generation progestins with progesterone-like properties, developed as oral contraceptives with excellent bioavailability and safety profile. We investigated the neuroprotective properties of these progestins *in vivo* using transient middle cerebral artery occlusion (MCAO) and *in vitro* using an oxygen-glucose deprivation and reoxygenation (OGD/R) model in primary neuronal cells.

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Conclusions. For the first time, here we delineate a potential role of desogestrel and drospirenone, both clinically approved and safe drugs in mitigating the consequences of stroke. Contraception with desogestrel and drospirenone in progestin-only preparations may be particularly beneficial for women at risk of stroke.

Keywords: $GABA_A$ receptors, desogestrel, drospirenone, neuroprotection, stroke

Translational Perspective

During their reproductive years, women have a lower mortality from cardiovascular disease than men do. This sex-associated benefit is most likely due to protection by endogenous sex hormones, among them, progesterone. Progestins are synthetically produced steroid hormones similar to natural progesterone, but with a more favorable bioavailability, predominantly used for contraception ("mini pill"). In our study, we demonstrate for the first time that progestins protect against experimental stroke-induced brain injury and functional deficits and suggest that strengthening of GABAergic signaling may mediate this response. Thus, progestins, already in use as oral contraceptives by millions of women, may be promising novel candidates for stroke treatment.

Introduction

The worldwide prevalence of stroke is significantly higher in women than in men (1). While premenopausal women are less affected by stroke than men of the same age, this advantage disappears after menopause when the incidence of cerebrovascular disease rises steadily (2). This may not only be the result of older age but also of lower endogenous estrogen and progesterone production in postmenopausal women (3). Accordingly, experimental and clinical data have documented that estrogen and progesterone mitigate brain damage after cardiovascular disease (4,5). Progestins are synthetically produced steroid hormones. Desogestrel, a 19-nortestosterone derivative, and drospirenone, a spironolactone derivate, are widely used in hormonal contraceptives (6). These preparations provide highly efficient and well tolerated contraception (7). Interestingly, desogestrel has also been found to reduce migraine frequency and intensity (8). In light of the pathophysiological parallels between certain types of migraine and ischemia and the higher incidence of stroke in migraineurs, this observation suggests a potentially protective effect of desogestrel in the context of stroke. This is further supported by studies demonstrating protection and enhanced regeneration afforded by the natural steroid hormone progesterone in different models of brain and cardiovascular diseases (9,10). In animal models of cerebral ischemia, progesterone has been shown to reduce lesion volume and improve functional outcome (11). Progesterone appears to stabilize the blood brain barrier (BBB), reduce cerebral edema, down-regulate inflammatory cascades, and decrease apoptosis in neurons (12). All these actions are plausible mechanisms for progesterone's neuroprotective effects. In addition to these effects through non-receptor pathways, progesterone elicits neuroprotective properties by modulating progesterone receptors and enhancing the activity of $GABA_AR$ via its metabolite allopregnanolone (13). $GABA_A$ exerts an inhibitory tone on glutamate mediated excitotoxicity after stroke (14). However, low bioavailability and rapid clearance of progesterones have complicated the applicability (15).

Our goal was to analyze a potential neuroprotective effect of the progestins desogestrel and drospirenone in experimental stroke. To address sex-specific differences in the response to progestin treatment, we analyzed male, female ovariectomized and female daily progestin-treated mice, the latter modeling women on progestin-only contraceptive pills (POP).

Methods

For the complete details of the methods used, see the Supplementary material online.

Experimental design

All experiments were performed in accordance with the guidelines and regulations approved by the Federal Veterinary Office of Switzerland (Veterinary Office of the Canton of Zurich), animal welfare assurance number ZH080/15.

We used male, female (12 weeks, Charles River, Margate, UK) and ovariectomized female C57/BL6 mice (12 weeks, Envigo, Horst, Netherlands). The experimental setup is shown in Figure 1. Desogestrel (1.5 mg/kg) and drospirenone (4 mg/kg) were injected intraperitoneally (i.p.). Further details regarding the preparation and administration of the treatments are available in the Supplementary material online.

Middle cerebral artery occlusion (MCAO)

Cerebral ischemia was induced by endovascular occlusion of the left middle cerebral artery (MCAO) for 60 min (detailed in Supplementary material online). All mice were weighed before MCAO and daily until day 3 post-stroke as an indicator of general well-being. A total of 203 mice were used in the study. Details regarding the number of animals per group, exclusion criteria and mortality are provided in Table 1. For infarct volume quantification, brain sections were stained with triphenyltetrazolium chloride (TTC).

Sensorimotor testing

Sensorimotor function was assessed using the adhesive tape removal test (16) as well as a composite observational neurological score (17) before MCAO and on days 1 and 3 (see also Supplementary material online).

Western blot analysis of $GABA_A$ receptor subunits

Twenty-two male mice were used for this analysis. Details on Western blotting are described in the Supplemental material online.

Cell culture and oxygen-glucose deprivation/reperfusion (OGD/R)

Primary mouse neural cells were obtained from Lonza (E14, 15 Mouse Cortex M-Cx-300; Lonza, Basel, Switzerland). Cell culture, oxygen glucose deprivation and cell viability assay are described in the Supplemental material online in detail. For dose-finding experiments, cells were treated with increasing concentrations of desogestrel and drospirenone (0.01, 0.1, 1, 10 µM) similar to previous cell culture experiments (18).

The role of $GABA_A$ receptors in progestin-induced neuroprotection against OGD/R injury was studied using picrotoxin (100 µM), a $GABA_A$ receptor antagonist. In separate experiments, we confirmed that the used dose of picrotoxin is not toxic for the primary neural cells used.

Twenty-four hours after OGD/R, cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Statistical analysis

Information about statistical analyses are provided in the Supplementary material online. The neurological scores are expressed as median ± interquartile range (IQR). All other data are mean ± SD; with differences $p < 0.05$ considered significant.

Results

Progestin treatment results in less sensorimotor deficits after MCAO

An overview of the treatment schedule is shown in *Figure 1*. Desogestrel and drospirenone treatment did not affect weight following MCAO. Mice in all treatment groups lost 10-20% of their pre-surgical body weight following MCAO (*Figure 2A, C, E*).

Male mice showed the severest functional impairment after stroke as well as the most pronounced preservation of functional deficits in response to progestin treatment (*Figure 2B*). In the sticky tape test assessing sensorimotor function of the affected forelimb, the latency to remove the sticky tape was lower at both D1 and D3 after treatment with desogestrel and drospirenone, compared to vehicle-treated mice. Female mice treated with one of the progestins also showed a better performance in the sticky tape test compared to vehicle treated mice, however; the effect was less than in males (*Figure 2D,F*).

Similar results were obtained using the 13-point neurological score: male mice with desogestrel or drospirenone treatment performed better at D1 and D3 post-MCAO than mice with vehicle treatment (*Figure 3A*). In addition, application of desogestrel and drospirenone in female ovariectomized (*Figure 3B*) and female daily-pretreated mice (*Figure 3C*) resulted in better neurological scores at D1 and D3.

Infarct volumes are lower with progestin treatment

Figure 3D shows representative brain slices stained with TTC at D3 after MCAO in vehicle, desogestrel and drospirenone treated mice. The lesion was largely confined to the cortical and striatal region of the brain. In the progestin-treated males, female ovariectomized and female non-ovariectomized daily-treated mice, infarct volumes were smaller than in vehicle-treated mice. Largest lesion volumes after cerebral ischemia were observed in male vehicle treated

mice (80.7 ± 18.6 mm^3, *Figure 3E*). After administration of desogestrel, and drospirenone, the damage was lower by about 71% and 54%, respectively.

In female ovariectomized mice treated with vehicle, average infarct volume was 69.4 ± 19.1 mm^3. Treatment with desogestrel and drospirenone resulted in smaller infarcts (about 48% and 61%, *Figure 3F*). Lesion volume was smallest in female non-ovariectomized vehicle-treated mice (57.2 ± 11.8 mm^3), supporting a protective role of endogenous estrogens and progesterone in these non-ovariectomized females. Accordingly, the protective effect of the therapeutically administered progestins on infarct volume in these female mice was less prominent, but still significant with lower infarct volumes by 51% (desogestrel) and 48% (drospirenone; *Figure 3G*).

Protein levels of $GABA_AR$ α4, and δ subtypes are higher after progestin treatment

Neurosteroids interact with certain subtypes of $GABA_AR$, regulating tonic inhibition and neuronal excitability in the ovarian cycle. Periodic cycling in $GABA_A$-R configuration and function in response to progesterone may underlie certain medical conditions that fluctuate during the ovarian cycle, such as catamenial epilepsy or migraine (19). In order to assess if similar regulatory pathways are involved in the progestin-induced protection after stroke, we analyzed brain $GABA_A$-R α4, α5 and δ subunit expression with different treatments by Western blot (*Figure 4*). Protein levels of the α4 and δ $GABA_A$-R subunits with desogestrel treatment were higher. Drospirenone also increased the levels of α4 on the ipsilateral side and δ on the contralateral side, but did not change expression of α4 on the contralateral and δ on the ipsilateral side. We did not find significant alterations in $GABA_AR$ α5 protein expression in response to progestin treatment.

Desogestrel- and drospirenone-induced protection from OGD/R *in vitro* requires $GABA_AR$ signaling

In order to determine whether progestin-afforded neuroprotection was mediated through the $GABA_AR$, we combined desogestrel and drospirenone with picrotoxin, a blocker of $GABA_AR$-coupled chloride channels, at a concentration of 100 μM. At this concentration, picrotoxin treatment was not toxic to non-OGD cells as judged by the MTT assays (*Figure 5A*). Furthermore, picrotoxin alone at 100 μM did not significantly affect the amount of cell death following OGD/R (Figure 5B). We then sought to establish the concentrations of desogestrel and drospirenone that would best reduce cell death following OGD/R. We found that 0.01 μM of drospirenone and 10 μM of desogestrel were the doses with the most pronounced effect (data not shown). In the vehicle-treated 120 min OGD/R cultures, cell viability was lower compared to non-OGD/R controls (*Figure 5B*). Pretreatment of the cultured cells with desogestrel and drospirenone resulted in higher cell viability after OGD/R (*Figure 5B*). However, desogestrel and drospirenone were no longer effective in the presence of picrotoxin (*Figure 5B*).

$GABA_A$ receptor blockage reduces progestin-induced neuroprotection after MCAO *in vivo*

To test whether mitigation of stroke-induced brain injury by desogestrel and drospirenone required $GABA_AR$ signaling *in vivo*, we added picrotoxin (0.1 mg/kg) to desogestrel and drospirenone treatment after MCAO in a separate group of male mice. Vehicle-treated mice had a long latency to remove the sticky tape (*Figure 6A*), low neurological scores at D1 and D3 (*Figure 6B*) and large lesion volumes after stroke (*Figure 6C*). Functional impairment was less in progestin-treated mice (*Figure 6A and B*). Infarct volumes, which were obtained from D3 or D7 TTC staining (*Table 1, Supplemental data*), were lower in the desogestrel- and drospirenone-treated groups (*Figure 6C*). Co-treatment with picrotoxin abolished the protection elicited by either desogestrel or drospirenone on functional readouts as well as lesion volume.

Discussion

Beyond recanalization in the acute phase, no other pharmacological intervention has demonstrated to mitigate brain injury in stroke patients. Here we report that the progestins desogestrel and drospirenone induce marked protection from neuronal injury in cell culture and *in vivo* models of cerebral ischemia, both in male and female mice. We observed tissue protection as well as preservation of sensorimotor function with progestin treatment. Efforts at blocking $GABA_AR$ signaling abolished neuroprotection in both model systems. Increased levels of the $GABA_AR$ subtypes α4 and δ were found particularly with desogestrel, but also with drospirenone treatment, raising the possibility that strengthening of $GABA_A$ mediated tonic inhibition might be involved in prevention of ischemic damage by the progestins.

Although neuroprotection was observed in all groups of animals, our data suggest additional endogenous protection elicited by female sex hormones (9). While male mice were most affected by stroke, female premenopausal mice with sustained basic ovarian hormone production had the smallest lesions and least functional impairment. However, even in this group, which received progestin-pretreatment suppressing ovarian hormone production similar to women using POP, there was a benefit of progestin-pretreated mice compared to sham-treated controls in stroke. The effect size was > 50% for infarct reduction with both progestins in this model, which is similar to previously reported progesterone-mediated effects in mice (11).

Despite a large number of experimental preclinical work documenting the neuroprotective effects of progesterone in neurological disease (11,20), two recently completed phase III clinical trials found that progesterone was not superior to placebo in improving outcomes after moderate to severe traumatic brain injury (15,21). As possible reasons for this failure, patient selection procedures and trial design as well as dose levels and/or durations of treatment have been discussed (22). Particularly, because of the low bioavailability and rapid clearance of exogenous progesterone, it is difficult to reach stable and effective plasma concentrations (22).

Desogestrel and drospirenone are third and fourth-generation progestins used as contraceptives by about 100 million women worldwide (6). While combined oral contraceptives containing estrogen and progestins increase the risk of thromboembolic complications, desogestrel-containing POP are not associated with an increase in cardiovascular risk and can safely be used in women with additional risk factors like advanced age, smoking, hypertension, or migraine (23). Currently, desogestrel is the only progestin available as a highly effective POP and therefore the more interesting candidate for future clinical studies, while a POP with drospirenone will be available in the near future (24). Compared to progesterone, both progestins have a well-characterized, favorable pharmacokinetic profile, including a longer half-life: drospirenone (31-32.5 h), desogestrel (8-32 h) versus progesterone (5-20 min) (25), no sedative side effects and higher bioavailability in the range of 62–76% (25). While effects of drospirenone on physiological or pathological conditions of the brain have not been evaluated, recent studies have shown a positive impact of desogestrel on migraine frequency and intensity in women (23,24).

Our study identifies protective properties of these progestins in models of stroke. Based on our results, for women taking desogestrel and drospirenone as contraceptives in POPs, an additional stroke-protective effect may arise, which would be particularly relevant for women at high risk of cerebrovascular disease (26). In order to characterize potential signal mediators of the observed progestin-induced neuroprotection in our study, we analyzed changes in the expression of $GABA_AR$ subtypes. Furthermore, we sought to inhibit $GABA_AR$ activity with picrotoxin both *in vivo* and *in vitro*. $GABA_AR$ play an important role in synaptic inhibition in the brain; mediating a tonic inhibition that lowers shunts excitatory currents and impairs activation of N-methyl-D-aspartate (NMDA) receptors (27). After stroke, there is an imbalance between inhibitory and excitatory neurotransmission, which creates an excessive release of glutamate as the pivotal event leading to cell death (14). There is sound evidence that enhancing GABAergic activity after cerebral ischemia is beneficial through inhibiting NMDA receptor-

mediated responses and therefore decreasing glutamatergic activity (14). The majority of post-stroke tonic neuronal inhibition is mediated by extrasynaptic $GABA_A$ receptors, predominantly containing α4, α5 and δ $GABA_AR$ subtypes (28,29). Progesterone acts on $GABA_AR$ through its metabolite allopregnanolone, which binds to discrete sites within the hydrophobic domain of the $GABA_AR$, resulting in potentiation of signaling activity (30–32). Progesterone may also have non-allosteric influences on the GABAA receptor by activating a signal transduction pathway, which in turn, influences GABA-gated currents through phosphorylation of discrete sites within certain subunits of the GABAA receptor (33).

In the present study, we show that the expression of $GABA_A$ α4 and δ receptor subunits is higher after application of the progestins desogestrel and drospirenone. While modulation of the $GABA_A$ α5 receptor subunit might also enhance post-stroke functional recovery (28), our data did not show changes in expression of this subunit after desogestrel or drospirenone treatment. Furthermore, the GABAA receptor expression and sensitivity to neurosteroids has been shown to be modulated by several factors, including age and gender (34,35). Indeed, several studies show significant age-related loss of the GABA neurotransmission (36–38). Furthermore, several neuroprotective molecules including the Clomethiazole (GABAA Modulator) act by delaying the ischemic cascade leading to cell death (39,40). Since the progestins act also through the GABAA receptors, they may be able to enhance GABAA function and act by leading to the inhibition on NMDA receptors and delaying the excitotoxic cascade of cell death after stroke.

In addition to potentiating GABA-ergic signaling, there are several other possible mechanisms, which might mediate beneficial actions of progestins in stroke. Although pharmacologically distinct, progestins are similar to progesterone, and several different pathways known to support the neuroprotective effect of progesterone in stroke may also be induced by the progestins, including neurotrophin expression or activation of the MAPK and Akt signaling pathways (41,42). Progesterone may act through novel receptor systems, such as the sigma receptor, to

activate intracellular protective signaling transduction pathways, reducing brain edema and BBB disruption (12). It is a free radical scavenger and may thereby mitigate oxidative stress-induced neuronal cell death (35). Furthermore, the progesterone metabolite allopregnanolone can act through the mitochondria by inhibiting the currents associated with opening of the mitochondrial permeability transition pore (mtPTP) (36). By this inhibition, allopregnanolone helps to reduce the potential apoptotic consequences of mtPTP opening (such as cytochrome c release) during stroke and may also elicit protective properties (36, 37).

Further studies are required to elucidate whether progestins establish protection through similar multiple pathways after stroke. Since we did not test later application time points after stroke or the combination of rtPA-thrombolysis with progestin-treatment, the applicability of desogestrel and drospirenone in these situations remains open.

In conclusion, despite progress in recanalization strategies, there is a pressing need to identify new treatments for the large fraction of stroke patients not eligible for recanalization or patients with deficits despite successful thrombolysis. In the last two decades, a large number of neuroprotective substances has been investigated in animal models of stroke, but translation from bench to bedside has remained difficult. We are convinced that the chances of applicability to stroke patients are high for desogestrel and drospirenone, as the treatment is efficient in males and females alike and the safety of these progestins has been proven (23). Therefore, the progestins desogestrel and drospirenone, already in clinical use as oral contraceptives, are promising candidates for therapeutic stroke applications.

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Conflict of Interest

GM is lecturer and member of the advisory board of Exeltis and lecturer for MSD. SW received a research grant from Boehringer Ingelheim and a speaker honorarium for Amgen outside the submitted work. MW reports grants and personal fees from Roche, Abbvie, Novocure, grants from Merck EMD, Piqur, OGD2, Tragara, Actelion, Acceleron, Bayer and personal fees from Tocagen, Celgene, Orbus, Progenics outside the submitted work. AL reports personal fees from Bayer and from Amge outside the submitted work. The remaining authors have disclosed that they do not have any conflicts of interest in relation to this work.

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Legends

Figure 1. Experimental design. Timeline of the study in (A) male and female ovariectomized (OVX) and (B) female daily-treated mice. Only female non-ovariectomized mice received pre-treatment with progestins or shams, to model chronic intake of progestin-only contraceptive pills in women of childbearing age. Mice were subjected to stroke surgery and the treatments were administered at 1, 6 and 24 h post-MCAO. Only female non-ovariectomized mice were pre-treated daily during 10 days before MCAO. The sensorimotor testing was performed before MCAO and at D1 and D3 post-stroke. Mice were euthanized at the end of the study and brains were harvested for infarct volume analysis and Western blotting. In experiment 2, for $GABA_A$ receptor inhibition, picrotoxin (or vehicle) was added to progestin or vehicle treatment after MCAO in a separate group of male mice (C) as well as to progestin treatments of primary neuronal cells during OGD/R in *vitro (D).* Note that in (C), the observation time was extended to 7 days to gather additional information about the effects of treatments during the post-acute phase after stroke.

Figure 2. Body weight and sticky tape test. Body weights are presented as percentage change compared to values prior to MCAO. Treatments did not affect body weight in male (2A), female OVX (2C) and female non-ovariectomized mice (2E). (B, D, F). Assessment of sensorimotor function using the sticky tape test in vehicle, desogestrel and drospirenone treated mice. Latency to remove a sticky tape applied to the right (contralateral) forepaw is shown before MCAO and at D1 and D3 post-MCAO in male, n=11-12 (B), female OVX, n =10-13 (D) and female non-OVX, 13-15 (F) mice. Data are mean ± SD. *p<0.05, **p<0.01 and ***p<0.001 versus vehicle treated mice in ANOVA repeated measures analysis.

Figure 3. Neurological score and infarct volume. 13-point neurological score for sensorimotor function before MCAO and at D1 and D3 post-MCAO in the different treatment groups. (3A) male (n=11-12 per group), (3B) female OVX (n=10-13 per group) and (3C) female non-OVX mice (n=13-15 per group). Data are presented as median and IQR in box-and-whisker plot, *p<0.05, **p<0.01 and ***p<0.001 versus vehicle treated mice using Mann Whitney U test. (3D) Representative coronal sections demonstrate the ischemic infarct by the absence of TTC staining at D3 following MCAO in male mice. Infarct volumes (in mm^3) with the different treatments in males (n=5-6 per group, 3E), female OVX (n=10-13 per group, 3F) and female non-OVX (n=11-12 per group, 3G) mice at D3 post-MCAO. Data are mean ± SD, *p<0.05, **p<0.01 and ***p<0.001 versus vehicle treated mice in one-way ANOVA.

Figure 4. Expression of $GABA_AR$ α4, α5- and δ subunits after MCAO. (A) Representative Western blots from male mice brains showing the difference in protein levels of the three $GABA_A$ receptors subunits after progestin treatment in contralesional and ipsilesional hemispheres on D3 after MCAO in male mice. Beta-actin was used as a loading control. (B, C, D) Data represent the average and standard deviation from three experiments. Data are mean ± SD, n=5-6 per group*p<0.05 versus vehicle treated mice.

Figure 5. *In vitro* protection of primary mouse neuronal cells against oxygen glucose deprivation/reperfusion (OGD/R) with desogestrel and drospirenone is blocked by picrotoxin. (A) Evaluation of picrotoxin (100 µM) toxicity on non-OGD cells *in vitro* using the MTT assay. (B) Cell viability in OGD/R with progestin treatments with and without antagonizing the $GABA_AR$ using picrotoxin. After 13 days in culture, cells were incubated in glucose-free OGD-medium containing the indicated doses of both progestins (between 0.01 and 10 µM), or the same volume of vehicle (0.1 % DMSO) and exposed to hypoxia for 120min. Thereafter, the OGD medium containing the tested substances was exchanged for regular culture medium and

cells brought into their previous culture conditions in 95% O_2 and 5% CO_2. Control cells had an exchange in regular culture medium and were kept within the previous culture conditions. Data are mean ± SD, n=4-6 per group ##p<0.01 versus control group and *p<0.05 versus vehicle treated cells. Viability was expressed as percentage relative to control cells of each series of experiments. Data from control cells were scaled to the mean of control levels of all the experiments (100%).

Figure 6. The $GABA_AR$ antagonist picrotoxin blocks desogestrel and drospirenone- induced protection after MCAO *in vivo*. Latency to remove a tape on the right (affected) forepaw in sticky tape removal test (A), neurological score (B), and infarct volume (C). Infarct volume was analyzed either at D3 or D7 (see table 1), therefore overall infarct size is less than in the previous experiments (Figure 3E). However, even with assessment until D7, treatments with both progestins significantly reduced infarct volume. Data are mean ± SD for A) and C) and median (IQR) for B), n=5-7 per group*p<0.05; **p<0.01 and ***p<0.001 versus vehicle treated mice.

Table 1. Details regarding the number of animals per group, exclusion criteria and mortality. Experiment 1: male, female and ovariectomized female mice were treated with vehicle, desogestrel or drospirenone. Sensorimotor testing was performed over three days and mice were sacrificed for TTC infarct volume staining at day 3. “Only behavior”: because of a technical problem with TTC staining in these eight mice, only behavioral analysis was realized. In experiment 2, male mice were treated with vehicle, desogestrel or drospirenone combined with either picrotoxin or vehicle. In this experiment, we had planned to extend the observation period to 7 days. However, due to substantial mortality within the 7 days particularly in the vehicle treatment group, for the sake of animal welfare, we later remained with the observation time of 3 days. Therefore, the TTC staining in this experiment 2 is a mixed readout from d3 and d7.